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SPR4-peptide Alters Bone Metabolism of Normal and HYP Mice

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Abstract

Context—ASARM-peptides are substrates and ligands for PHEX, the gene responsible for X-linked hypophosphatemic rickets (HYP). PHEX binds to the DMP1-ASARM-motif to form a trimeric-complex with $\alpha_5\beta_3$ -integrin on the osteocyte surface and this suppresses FGF23 expression. ASARM-peptide disruption of this complex increases FGF23 expression. We used a 4.2 kDa peptide (SPR4) that binds to ASARM-peptide and ASARM-motif to study DMP1-PHEX interactions and to assess SPR4 for treating inherited hypophosphatemic rickets.

Design—Subcutaneously transplanted osmotic pumps were used to infuse SPR4-peptide or vehicle into wild-type mice (WT) and HYP-mice for 4 weeks.

Results—Asymmetrically distributed mineralization defects occurred with WT-SPR4 femurs. Specifically, SPR4 induced negative effects on trabecular bone and increased bone volume and mineralization in cortical-bone. Markedly increased sclerostin and reduced active β -catenin occurred with HYP mice. SPR4-infusion suppressed sclerostin and increased active β -catenin in WT and HYP mice and improved HYP-mice trabecular mineralization defects but not cortical mineralization defects.

Conclusions—SPR4-peptide has bimodal activity and acts by: (1) preventing DMP1 binding to PHEX and (2) sequestering an inhibitor of DMP1-PHEX binding, ASARM-peptide. In PHEX defective HYP-mice the second pathway predominates. Although SPR4-peptide improved trabecular calcification defects, decreased sclerostin and increased active β -catenin it did not correct HYP-mice cortical mineralization defects on a normal phosphate diet. Thus, for inherited hypophosphatemic rickets patients on a *normal phosphate* diet, SPR4-peptide is not a useful therapeutic.

Keywords

Rickets; MEPE; DMP1; FGF23; PHEX; Sclerostin

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Introduction

Matrix-Extracellular-Phospho-Glycoprotein with ASARM-motif (MEPE) (1), is a major bone-matrix protein that regulates bone-mass (1–15). Genome wide association studies (GWA) also confirm MEPE is a major gene locus for bone mineral density and osteoporosis (16–23). Serum levels correlate with serum phosphorus, parathyroid hormone and bone mineral density (BMD) (6, 24). Notably, bone mass and trabecular content increase as MEPE null-mice age and decrease in mice over expressing MEPE (11, 15). In these studies we used mice with a defect in the PHEX gene (HYP-mice) (25) that have increased expression of MEPE and ASARM-peptides (2, 5, 6, 8–10, 12–14, 26). These mice are murine homologues for X-linked hypophosphatemic rickets (HYP) and are also hyperglycemic, hypoinsulinemic with altered energy metabolism (27–30). The physiological substrate for PHEX is free ASARM-peptide or the ASARM-motif and the ASARM-motif occurs in a group of extracellular matrix-proteins called SIBLINGs (26). These SIBLINGs include DMP1, MEPE, DSPP, Osteopontin, Statherin and BSP (26). Mutations in DMP1 are responsible for autosomal recessive hypophosphatemic rickets (ARHR-1) a disease that has an overlapping pathophysiology with HYP (31, 32). In both ARHR-1 and HYP increased circulating ASARM-peptides contribute to the mineralization defect and hyperosteoridosis (2–4, 6, 8, 10, 12–14, 33–35). Compelling evidence shows that on the external surface of the osteocyte, PHEX binds to the DMP1 ASARM-motif to form a trimeric complex with $\alpha_5\beta_3$ -integrin that suppresses FGF23 expression (26). To study integrin, PHEX, DMP1 and ASARM-peptide interactions we designed a small 4.2 kDa PHEX related peptide (SPR4-peptide). This peptide specifically binds to both the ASARM-peptide and ASARM-motif (9, 12, 30). Also, SPR4-peptide has potent biological effects, inhibits PHEX and neutralizes ASARM-peptide, an inhibitor of mineralization (9, 12, 30). Recently, we showed infusion of SPR4-peptide corrects energy metabolism defects in HYP-mice and improves energy metabolism in Wild Type mice (30).

The present study extends our previous work and here we describe the effects of infusing SPR4-peptide on bone mineral metabolism in wild type (WT) and HYP mice. The femurs of WT-SPR4 mice developed an uneven mineralization distribution along the bone-shaft. Specifically, increased BV/TV (bone-volume/ tissue-volume) and mineral content with reduced cortical-osteoid occurred in WT-SPR4 mice. In contrast an increased osteoid content occurred across the metaphysis and epiphysis of WT-SPR4 mice. Compared with WT-mice, HYP-mice had markedly increased sclerostin in bone, kidney and circulation. SPR4-infusion suppressed sclerostin and increased active β -catenin in both WT and HYP mice but did not correct the HYP mice cortical bone mineralization defects. A significant but partial correction of the HYP-SPR4 mice trabecular defect did occur.

This study shows SPR4-peptide infusion has pleiotropic effects on bone and phosphate mineral metabolism. Consistent with this, the four-week infusion partly improved mineral metabolism but also induced bone-mineral abnormalities in WT mice that mirrored the HYP phenotype. The pleiotropic nature of SPR4-peptide likely reflects its ability to bind with the DMP1 ASARM-motif and free ASARM-peptide. This dual ability will either result in: (1) competitive prevention of PHEX-DMP1-integrin binding and thus altered FGF23 expression; or (2) the direct binding of the PHEX substrate, ASARM-peptide. In conclusion,

SPR4-peptide suppresses sclerostin, neutralizes ASARM-peptides, increases active β -Catenin and partially improves bone metabolism. On a normal phosphate diet, SPR4-peptide infusion is not a useful therapeutic for treating inherited X-linked hypophosphatemic rickets.

Methods

Animals, Procedures, Diets and Peptides

Ethics Statement—Mice (C57BL/6) were housed at the University of Kansas Medical Center under the supervision of the Department of Laboratory Animal Resources. The policies and procedures of the animal laboratory are in accordance with those detailed in the Guide for the Care and Use of Laboratory Animals published by the US Department of Health and Human Services (DHHS Publ. NIH 86-23, 1985). Procedural protocols were approved by the University of Kansas Institutional Animal Care and Use Committee.

Procedures, Diets and Peptides—Male (5-week) C57B/L6 mice that were wild-type (WT) or mutant X-linked Hypophosphatemic Rickets mice (HYP) were used for the study. All mice were maintained on a 1% phosphorus and 2.4IU/g Vitamin-D3 diet (Harlan Teklad Rodent Diet 8604, Indianapolis, IN). Mice (5 week) were surgically implanted with Alzet osmotic pumps (Durect Corporation, Cupertino, CA) as described previously (8, 30) and infused with SPR4-peptide (276 nmoles/hr/kg) or vehicle (0.9% physiological Saline; VE) for 28 days. Specifically, Alzet pump model #2004 with a constant infusion rate of 0.25 μ L/h over 28 days was used. Four groups were studied (n=6/group) as follows: (1) Wild type mice infused with vehicle (WT-VE); (2) HYP-mice infused with vehicle (HYP-VE); (3) Wild type mice infused with SPR4 peptide (WT-SPR4); and (4) HYP-mice infused with SPR4-peptide (HYP-SPR4). The SPR4-peptide (4.2-kDa) (NH₂-TVNAFYASTNYPRSLSYGAIGVIVGHEFTHGFDNNGRGENIADNG-OH) was synthesized using standard techniques by Polypeptide Laboratories (San Diego, CA 92126) as described previously (9, 12, 30). Peptide purity was greater than 80% via HPLC, ion-exchange and also mass spectrometry. SPR4-peptide was dissolved as follows; 100 μ L/1 mg of peptide of 25 mM acetic acid was first added to dissolve the peptide, then 900 μ L of 50 mM Tris pH7.4/150 mM NaCl was added and after thorough mixing 20 μ L of 1 mM ZnCl₂. Note, it is important to add the ZnCl₂ last to maintain peptide solubility. The final buffer composition was 44 mM Tris pH7.4/132 mM NaCl/19.6 μ M ZnCl₂.

Serum and Urine Analysis

At specific intervals throughout the experiment as detailed in the results section tail blood-samples were collected in serum-separator tubes and serum prepared as described previously (8, 30). On the final day of the infusion experiment (day 28) blood and urine was collected from mice fasted overnight in metabolic cages with full access to water (1 cage/mouse). The blood from the final bleed was collected by cardiac exsanguination and serum urinalysis carried out as described previously (5, 8, 11, 13, 14, 30). Briefly, Osteocalcin (Mouse Osteocalcin EIA Kit; BTI, Stoughton, MA), alkaline phosphatase (Liquid Alkaline Phosphatase; Pointe Scientific Inc, Canton, MI), 1,25 (OH)₂D₃ (IDS Inc., Fountain Hills, AZ) and FGF23 (Kainos Laboratories Inc., Tokyo, Japan) were measured on serum samples. Inorganic phosphorus, calcium, creatinine (Pointe Scientific Inc, Canton, MI) and

Osteopontin (Quantikine Mouse Osteopontin; R&D Systems, Minneapolis, MN) levels were assessed both in serum and urine. A competitive ELISA kit was used for the ASARM peptide measurement as previously published for serum and urine samples (6, 8, 11, 30).

Bone Analysis: micro computed tomography (μ CT) & Dual X-Ray Absorptiometry (DEXA)

Dual X-Ray Absorptiometry (DEXA) using a PIXImus system (LUNAR Corporation, Madison, WI) and micro computed tomography (μ CT) using a Scanco μ CT 40 system was carried out as described previously (11, 13, 30). Mice bones and kidneys (fixed and ethanol dehydrated) were scanned with a high-resolution μ CT (μ CT40; Scanco Medical, Southeastern, PA) as previously described (11, 13). Data were acquired at 55 KeV and 6 μ m cubic resolutions for isolated specimens. Three-dimensional reconstructions for bone samples were generated with the following parameters: Sigma, 0.8; Support, 1; Threshold, 250 (spongiosa) or 280 (cortex). Renal calcifications were assessed with a threshold of 200. Cortical thickness and tissue mineral density were calculated by integration of the value on each transverse section of a set of 150 chosen in the midshaft area. Tissue mineral density was derived from the linear attenuation coefficient of thresholded bone through precalibration of the apparatus for the acquisition voltage chosen. The bone volume fraction of trabecular metaphysis (VOX BV/TV) was measured on a set of 100 sections under the growth plate, within the secondary spongiosa. Trabecular thickness (Tb.Th), trabecular number (Tb.N), trabecular separation (Tb.Sp), and structure model index (SMI) were calculated without assuming a constant model, as previously described (11, 13). SMI estimates the plate-rod characteristics of a structure; its value is 0 for an ideal plate, and 3 for an ideal rod, with intermediate values reflecting the volume ratio between rods and plates. For the assessment of bone microstructure in rodents the guidelines detailed by Bouxsein et al 2010 (36) and standardized as described by Parfitt et al 1987 (37). A dual energy X-ray PIXImus densitometer (LUNAR Corporation, Madison, WI) was used for measuring bone mineral density (BMD). Four different sites were determined by adjusting the region of interest (ROI): entire humerus, entire femur, entire tibia and L1 to L5 vertebrae. ROI was adjusted on each bone length and width.

Bone Histology and Histomorphometry

Bone histology histomorphometry was carried out as described previously (11, 30). Specifically, bone growth was assessed by slide caliper measurement of bone length. Animals were labeled by intraperitoneal (ip) injection of 30 μ g/g tetracycline, 40 μ g/g alizarin complexone-dehydrate and 15 μ g/g calcein (Sigma-Aldrich Co, MO, USA) respectively on days 9, 6 and 3 prior to sacrifice. Bones were excised, fixed, dehydrated in ethanol, and embedded in methylmethacrylate at low temperature. Longitudinal coronal slices were cut with a Leica model RM2255 microtome (Leica Biosystems; Buffalo Grove, IL USA 60089) and used for modified Goldner staining, tartrate-resistant acid phosphatase (TRAcP), alkaline phosphatase (ALP) or left unstained. Trabecular bone volume (BV/TV), osteoid surface (OS/BS), thickness (O.Th) and volume (OV/BV) were measured on Goldner sections. Dynamic bone remodeling parameters were measured by histomorphometry on unstained sections for labeled surfaces (MS/BS), mineral apposition rate (MAR), and BFR (BFR/BS). These parameters were combined to assess osteoid mineralization through MLT ($MLT = [O.Th \times OS] / [MS \times MAR]$) and Osteoid Maturation Time ($OMT = O.Th / MAR$).

Osteoclast surfaces (Oc.S/BS) and numbers (N.Oc/B.Ar; NOc/BPm) were measured on tartrate resistant acid phosphatase (TRAcP) stained sections. Widths of proliferative and hypertrophic chondrocytes zones were measured at multiple sites along the growth plate. Non-decalcified histology following tetracycline labeling was used to assess bone histologically. Explora-Nova histomorphometric software (F17011 LaRochelle Cedex 1France) was used to analyze the microscopically imaged samples and the guidelines described by Ott 2008 (38) were used to calculate histomorphometric measurements of bone turnover, mineralization and volume. Bone histomorphometric nomenclature, symbols and units were standardized as described by Parfitt et al 1987(37).

Immunohistochemistry (IHC)

Immunohistochemistry was carried out as described previously (11, 12, 30). Specifically, proximal tibias and distal femurs were decalcified in 0.1M EDTA aqueous solution for 2 weeks until complete demineralization. Decalcified bones and left kidneys were dehydrated in absolute ethanol and embedded in paraffin. 5µm thick sections were cut on a rotary microtome. Sections were dried overnight on pre-charged pre-cleaned slides (VWR Scientific, PA, USA), deparaffinized and rehydrated. Osteoblastic and osteoclastic cell cultures were cultured as described previously on BD cell-culture treated bio slides and processed in the same manner as tissue sections (11, 12). For immunohistochemistry, after antigen retrieval by incubation in citric acid buffer 10mM pH 3 for 60min at 37°C, nonspecific sites were blocked with 1X animal free blocker (Vector Laboratories Inc., CA, USA) and then sections were incubated with specific primary antibodies for 1 hour. An immunohistological vectastain ABC kit (Vector Laboratories Inc., CA, USA) is routinely used and slides counterstained with DAPI or methyl-green, dehydrated and mounted with entellan. Polyclonal primary antibodies used are detailed in supplementary Table S1. The *in situ* hybridization was performed using a modification of the procedure previously described (6, 11, 12, 30). Probes were labeled with fluorescein tag using the FastTag Basic Labeling Kit and detected with an alkaline phosphatase anti-fluorescein (Vector Laboratories Inc., CA, USA) antibody according to the manufacturer's instructions.

RNA Isolation, tissue extraction, Real Time PCR analysis & Western Blotting analysis

The above methods were performed as described previously (8, 11, 12, 30) with specific polyclonal primary antibodies and primers shown in Tables S1 and S2 respectively. Transferrin was used as an internal reference for all protocols (RNA and protein) since our previous studies showed GAPDH is markedly suppressed in HYP-mice bone (-X 6.2) and kidney (-X 2.0) (30). The Pfaffl mathematical model for the relative quantification of real-time PCR data was used to measure relative gene expression (39).

Statistical analyses

Statistical analysis was performed using statistical software STATISTICA (StatSoft Inc., Tulsa, OK, USA) or PRISM5 (GraphPad Software inc., La Jolla, CA USA). Differences between groups were initially analyzed by two-way ANOVA. When F values for a given variable were found to be significant, the sequentially rejecting Bonferroni-Holm test was subsequently performed using the Holm's adjusted p values. For qRT-PCR gene analysis

fold differences in expression calculated by the Pfaffl method(39) were statistically analyzed for significance using the One Sample t-test and the Wilcoxon Signed rank-test with theoretical means set to 1. Results for all tests were considered to be significantly different at $p < 0.05$.

Results

HYP and WT mice bone-kidney marker mRNA expression and protein levels in serum and urine

We previously reported a detailed analysis of serum chemistry, urine chemistry, bone-renal mRNA expression and DEXA (Dual Energy X-ray Absorptiometry) fat mass analysis for both HYP mice and WT mice infused with SPR4 peptides (30). In this paper we have focused on the unreported bone phenotype findings. Specifically, Table 1 shows the additional urine and sera chemistries and Table 2 mRNA expression profiles. As expected significant increases in serum alkaline phosphatase, osteopontin and sclerostin occurred with HYP mice with no significant changes in serum klotho (Table 1A). Increased urinary osteopontin and markers of bone resorption (CTX and PYD) also occurred (Table 1B). Wild type and HYP mice treated with SPR4-peptide showed increases in circulating alkaline phosphatase and osteopontin with decreased sclerostin (Table 1A). The changes in osteopontin and sclerostin are consistent with mRNA expression profiles for bone and kidney (Table 2A and B) and also immunohistochemistry data (Figure S1). Of note, the increased sclerostin in HYP mice was accompanied by reduced active bone β -catenin (Figure 1). Also, the SPR4-peptide induced suppression of sclerostin in both HYP and WT mice resulted in increased active bone β -catenin (Figure 1).

SPR4-peptide induces asymmetric effects on HYP & WT bone (μ CT & dynamic/static histology)

The phenotypic changes in HYP mice bone is reported extensively in the literature (26). Our findings using histology, μ CT and DEXA is consistent with previous research. As expected HYP mice showed major defects in mineralization in both epiphyseal cancellous bone and cortical bone with severe hyperosteoidosis. The increased sclerostin with reduced active β -catenin (serum, mRNA and immunohistochemistry and western) occurred with HYP vehicle mice compared with WT vehicle mice (Figure 1 and Figure S1; Tables 1A and 2A). This would suggest “with the mineralization defects” that HYP mice osteoblasts are immature and unable to differentiate fully.

SPR4 peptide infusion induced different results for HYP and WT type mice. This likely reflected the ability of SPR4-peptide to bind to the DMP1 ASARM-motif and the free ASARM-peptide respectively. Specifically: (1) the competitive inhibition of PHEX binding to DMP1 in WT mice; and (2) the binding of the PHEX substrate (ASARM peptide) in the “PHEX-null” HYP mice. For both WT and HYP SPR4-treated mice a marked decrease in bone-volume (BV/TV) occurred at the top of the epiphysis, mid epiphysis and metaphysis as illustrated in Figure 2. In contrast, the diaphyseal region of WT SPR4 treated mice showed major increases in BV/TV whereas the diaphysis of HYP SPR4 treated mice remained significantly reduced (Figure 2). The CT BV/TV analyses shown in Figure 2 agreed with the

histology data (Figure 3 (A, B, C & D); Tables S3, S4, S5 and S6). With HYP-SPR4 mice, a significant decrease in the hydroxyapatite mineral content (HA mg cm⁻³) of the cortical envelope encompassing the epiphyseal region, metaphysis and diaphysis occurred (Figure 2 and Figure S2). In contrast, for WT-SPR4 mice, a significant increase in HA occurred for these regions (Figure 2 and Figure S2). Despite the increased HA and BV/TV in WT-SPR4 mice, a negative impact on μ CT cortical bone quality metrics occurred. Specifically, the cortical regions of SPR4 treated WT and HYP mice showed significantly reduced cortical areas (Ct.Ar), cortical thickness (Ct.Th), bone surface (BS), moment of inertia (MOI) (Figure 3 (C & D), and Figure S2; Table S5 and S6). In contrast, an increased bone-surface/bonevolume (BS/BV) occurred with SPR4 treated WT mice ("Figure 3D and Table S5"). For cancellous bone, histomorphometric analysis showed SPR4 treatment reduced femur BV/TV significantly in WT mice (Figure 3A, Table S3). Also, high resolution μ CT analysis confirmed a reduced trabecular BV/TV for both WT and HYP mice treated with SPR4 peptide (Figure 3 (A & B) and Figure 4; Tables S3 and S4). WT-SPR4 treated mice displayed reduced trabecular numbers with increased spacing (Figures 3 (A & B) and Figure 4; Tables S3 and S4). HYP SPR4 mice also showed significantly increased trabecular spacing, reduced trabecular thickness and reduced trabecular numbers (Figures 3 (A & B) and Figure 4; Tables S3 and S4). Consistent with this, SPR4-peptide treatment in WT mice induced a significant increase in bone resorption parameters as follows: (1) osteoclast numbers (Oc.N/BS); (2) erosion surfaces (EV, EV/BV and ES/BS); (3) tartrate resistant acid phosphatase (TRACP) activity (Figure 3A and Figure S3; Table S3); and (3) increased urinary C-terminal telopeptide (CTX) peptides (Table 1B). In contrast, although HYP-SPR4 mice did not show statistically different osteoclast numbers (Oc.S/BS%, Oc.N/Bs), a significant increase in bone-erosion metrics did occur with both WT-SPR4 and HYP-SPR4 mice as follows: (1) erosion volume and surfaces (EV and EV/BV); (2) bone TRACP activity; and (3) urinary CTX peptides (Figures 3A and Figure S3; Table 1B and Table S3). Thus, with WT mice the increased bone resorption induced by SPR4 peptide is likely the result of increased osteoclast numbers and activity. With HYP mice, the increased bone resorption is likely chiefly due to increased osteoclast activity and not numbers.

SPR4-peptide infusion: *increased* HYP-cancellous & WT-cortical; *impaired* HYP-cortical & WT-cancellous mineralization

SPR4 peptide infusion of WT type mice induced mineralization defects in cancellous bone consistent with inhibition of PHEX activity. Specifically, WT SPR4 mice showed increased osteoid volume (OV/BV); (2) increased osteoid surface (OS/BS); and (3) increased osteoblast surface (Ob.S/BS) for trabecular bone (Figures 3A and Figure S4; Table S3 and S7). In contrast, the cortical region of WT mice treated with SPR4- peptide showed: (1) decreased osteoid volume (OV/BV); (2) decreased cortical osteoid thickness (Ct.Th (osteoid)); (3) decreased cortical osteoid area (Ct.Ar (osteoid)); and (4) increased mineralization (Figures 3C and Figure S5; Table S5 and Table S6). In contrast to WT mice, HYP SPR4 infused mice showed improvements in trabecular mineralization defects consistent with sequestration of ASARM-peptide, a PHEX substrate and inhibitor of mineralization (minhibin). Specifically, a: (1) decreased osteoid-volume (OV/BV); (2) decreased osteoid-surface (OS/BS); (3) decreased osteoid-thickness (O.Th); and (4) decreased osteoblast surface (Ob.S/BS) occurred (Figures 3A and Figure S4; Table S3).

Both WT and HYP mice infused with SPR4 showed major increases in cortical indices for mineralization with reduced osteoid content (Figure 3C and Figure S5; Table S6). Specifically a reduction in: (1) osteoid cortical-area (Ct.Ar (Ost)); and (2) osteoid cortical-thickness (Ct.Th (Ost)). However, although WT SPR4 mice showed reduced cortical osteoid volume (OV/BV) no significant change occurred with HYP SPR4 treated mice (Figure 3C and Figure S5; Table S6). A marked improvement in canal number (Ca.N), canal area (Ca.Ar) and cortical porosity (Ct.Por) also occurred but femur length was reduced (Figures 3C, Figure S5 and Table S6).

DEXA Analysis: SPR4-peptide induces Static and Dynamic changes in BMD and BMC

Intriguingly, although as expected DEXA showed significant decreases in bone mineral density (BMD) and bone mineral content (BMC) for total body, femurs and vertebrae for HYP mice compared to WT mice (Figure 5A), we also observed striking dynamic abnormalities with HYP vehicle mice weight, femurs and vertebrae over the time course (4 weeks) of the experiment ("Figure 5 (B & C)" and "Table S8"). Specifically, a significant increase in the temporal percentage change of BMD and BMC occurred with HYP vehicle vertebrae and femurs over the 4 weeks compared to WT vehicle mice ("Figure 5C and Table S8"). Thus, a reduction in static BMC and BMD metrics occurred with HYP mice compared with wild type mice at all time-points (Figure 5A). In contrast an accelerated increase in both BMD and BMC occurred over the 4 weeks for HYP mice femurs and vertebrae ("Figure 5C and Table S8"). No significant difference occurred between HYP mice and WT mice *dynamic* total-body BMD and total-body BMC percentage change over 4 weeks ("Figure 5C and Table S8"). In summary, HYP mice as expected had major mineralization defects, growth plate abnormalities, reduced trabecular bone, with abnormal indices of bone formation and resorption. Although an increase in dynamic total-BMD occurred with HYP-mice treated with SPR4-peptide (Figure 5D and Table S8), an overall negative and static dynamic effect on BMD and BMC occurred for both WT-SPR4 and HYP-SPR4 mice femurs and vertebrae (Figure 5 (A, C & D); Table S8).

Discussion

The findings reported in this study support the ASARM-model in (26). They also extend our previous findings on the effects of SPR4-peptide infusion on energy and phosphate metabolism in wild type (WT) and HYP mice (30). Specifically, we designed this peptide (SPR4-peptide) for studies on PHEX and ASARM in WT and HYP mice and reported the effects in several studies (9, 12, 30). This study for the first time reports SPR4-peptide mediated changes in bone for both WT and HYP mice. SPR4-peptide is a small 4.2 kDa peptide incorporating the active catalytic and binding domains of PHEX (12). It binds specifically to the only known physiological substrates for PHEX, the ASARM-peptide and ASARM-motif (8, 9, 12, 14, 30, 33–35). We previously showed SPR4 effects serum phosphorus levels differently in WT and HYP mice (30). In WT mice with active PHEX, SPR4 induces hypophosphatemia, increases FGF23 and PTH expression. A compensatory decrease in KLOTHO, an FGF23 coactivator with increased $1.25(\text{OH})_2\text{D}_3$ and hypercalcemia also occurs. In contrast, a partial improvement in HYP-mice hypophosphatemia occurred without changes in FGF23 or PTH. The hypophosphatemia in

WT-mice likely occurs because of SPR4 inhibition of PHEX coupled with compensatory changes in KLOTHO and PTH expression (30). Since HYP mice do not have active PHEX, their improved serum phosphorus occurs by SPR4 neutralization of ASARM-peptide, a molecule that can induce hypophosphatemia independent of FGF23 and PTH (5, 8, 12–14, 30, 40–42).

We and others have reported increased sclerostin in HYP-mice bone, kidney and circulation (9, 30, 43). Also, adding sclerostin to human trabecular bone or osteoblast cultures induces increased osteocyte lacunar area, MEPE expression and mineral dissolution (9, 44). In this study we show the increased sclerostin accompanies a decrease in active bone β -catenin. In further support of our findings a report by Farrow et al 2010 showed suppressed Wnt/ β catenin signaling in HYP mice. Notably, increased phosphorylated β Catenin (p- β -Cat) occurred in the distal collecting duct of HYP mice kidneys (43). Phosphorylated β Catenin (p- β -Cat) is a well-characterized marker of “suppression” of Wnt signaling and their findings agree with our p- β -Cat observations on the whole kidney (Figure 1). Several studies confirm a major role for β -catenin in mineralization (45–48). For example, mice lacking osteoblast β -catenin are osteopenic with increased osteoclasts, increased bone resorption and reduced mineralization. These mice also produce increase levels of RANKL (Receptor-Activated-by-Nuclear-Factor- κ B-Ligand) an osteoclast differentiation-factor, with delayed and reduced expression of osteocalcin, markedly reduced mineralization and severe osteopenia (47). These compound changes mirror the abnormalities we measured in HYP-mice that have reduced levels of β -catenin. Thus, suppressed active β -catenin and increased sclerostin is consistent with HYP-mice hypomineralization.

SPR4-peptide infusion of WT mice (WT-SPR4) induced changes that mimicked HYP-mice. For example, like HYP-VE mice, WT-SPR4 mice showed increased bone-resorption, reduced leptin, adiponectin, sympathetic-tone and increased CTX-collagen-peptides (marker of bone resorption) as well as hypophosphatemia, increased FGF23 and PTH (30). In contrast, SPR4 treatment dramatically reversed and corrected the increased sclerostin levels and gene expression in HYP mice kidneys and bone (30). Also, consistent with this a commensurate increase in active β -catenin occurred (Figure 1). Wild type mice treated with SPR4 also showed a major decrease in serum, bone and renal sclerostin with increased active β -catenin. Since HYP mice lack active PHEX protein, we infer SPR4-peptide induces changes in both WT-SPR4 and HYP-SPR4 mice by binding and neutralizing ASARM-peptide. Consistent with this interpretation, cortical bone of both WT-SPR4 and HYP-SPR4 mice showed increased mineralization and reduced osteoid content. In contrast, only partial improvements in mineralization occurred with HYP-SPR4 cancellous bone and the trabecular bone of WT-SPR4 mice showed increased osteoid content. Thus, infusing WT-mice cancellous bone with SPR4-peptide resulted in a coupled suppression of PHEX (a protein needed for mineralization) and sequestration of ASARM-peptide (a mineralization inhibitor). The net effect resulting in increased osteoid because of skewed PHEX inhibition in WT-SPR4 cancellous bone but not cortical bone. In contrast, with HYP-SPR4 mice the chief effect of SPR4 infusion was likely binding of excess free ASARM-peptide (mineralization inhibitor) and thus improved mineralization since these mice lack active PHEX. This model is illustrated schematically in Figure 6. Of note, uric acid levels in HYP mice are significantly lower than WT-mice (30) and uric acid levels correlate positively with

bone health and osteoporosis (49–51). Also, SPR4-peptide increased uric acid levels in WT-mice but not HYP-mice suggesting a role for PHEX (30). Of relevance to the bone phenotypes of HYP-mice and mice infused with SPR4-peptide, we previously reported altered expression of FAM20C, an ASARM specific bone kinase (30). Deletion of bone FAM20C in mice results in autosomal hypophosphatemic rickets (ARHR2) (52–55). Notably, our previous study showed infusion of SPR4-peptide in both WT and HYP mice markedly suppressed expression (30). This might be responsible for the uneven bone mineralization phenotypes we found with the SPR4-peptide infused mice in this study.

In conclusion, SPR4 treatment of WT-mice induced similar abnormal changes found in HYP-mice bone but with key differences. These differences were asymmetric and involved negative effects on epiphyseal cancellous-bone and positive effects on cortical-bone (BV/TV and mineralization). These asymmetric changes are likely attributable to active PHEX in WT mice and a combination of SPR4-peptide suppression and enhancement of PHEX activity. Specifically, SPR4-peptide infusion induces pleiotropic pharmacological effects on bone and mineral homeostasis that are consistent with the ASARM-model (Figure 6). We hypothesize these pleiotropic effects occur in wild type mice by: (1) the competitive prevention of PHEX binding to the DMP1- ASARM motif by SPR4-peptide (SPR4 binds to the DMP1 ASARM-motif). This disrupts the [DMP1-PHEX- $\alpha 5\beta 3$ -integrin] trimeric complex thereby increasing FGF23 expression and reproducing key phenotypic changes seen in HYP-mice; and (2) enhancing PHEX activity in WT-mice by binding to an endogenous inhibitor of PHEX, ASARM-peptide. This stabilizes the [DMP1-PHEX- $\alpha 5\beta 3$ -integrin] trimeric complex, decreases FGF23 expression and improves bone metabolism. Also, SPR4 partially suppresses the pathological effects of increased ASARM-peptide levels that occur in HYP-mice by binding to free ASARM-peptide. Although SPR4-peptide partially improved trabecular calcification defects, decreased sclerostin and increased active β -catenin it did not correct HYP-mice cortical mineralization defects in animals fed a normal phosphate diet. Therefore, we infer that for a normal phosphate diet, SPR4-peptide is not a useful adjuvant for treating X-linked hypophosphatemic rickets.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- We examine *in vivo* bone effects of SPR4-peptide; a ligand for both PHEX substrate and ligand (ASARM-motif and ASARM-peptide).
- SPR4-peptide suppressed sclerostin and increased active β -catenin in wild type (WT) and HYP mice.
- SPR4-peptide increased cortical-bone-volume and mineralization but inhibited WT trabecular bone.
- SPR4-peptide improved HYP-mice trabecular but not cortical mineralization defects.
- On a normal phosphate diet SPR4-peptide is not a useful adjuvant for treating X-linked rickets.

Figure 1.

Bone (femur) active β -catenin protein is suppressed in HYP mice and SPR4 infusion increases active β -catenin in both HYP and wild type mice. Western analysis of protein lysates prepared from femurs frozen in liquid nitrogen were undertaken as described in methods and published previously (8, 11, 12). Each lane was loaded with same amount of protein for each sample. Transferrin was used as an internal control for chemiluminescent pixel density calculations (BioRad Qty1 software and FluorS MaX Imaging). One way Anova and Newman-Keuls Post Test was used to calculate statistical significance ($P < 0.01$ * and $P < 0.001$ **).

Figure 2.

Regional percentage changes in femur bone-volume and hydroxyapatite content as measured by μ CT (vehicle versus SPR4-peptide). For additional information, absolute numbers and statistics on the cancellous and cortical regions see also Figures 3 (A, B, C & D) and supplementary Tables S3, S4, S5 & S6). Mice were sacrificed on day 28 and femurs collected for analysis as described in methods. The upper left quadrant of the figure contains a cartoon illustration of the distal femur with reference points that code for the regional measurements depicted on the histograms (1. top-distal epiphysis, 2. mid-distal epiphysis, 3. metaphysis and 4. diaphysis). The histogram bars represent percentage changes of SPR4-infused mice relative to vehicle-infused mice with vehicle normalized to baseline (0). Values are means of percentage difference and are significant ($* = P < 0.05$) unless indicated by NS (unpaired t test confidence interval 95%). Histogram bars greater than zero indicate an increase in SPR4 treated mice relative to vehicle and a decrease for negative values. **Index:**

% BV/TV = mean percentage change in bone volume/total volume of SPR4-infused mice relative to vehicle infused mice; **% HA** = mean percentage change in bone hydroxyapatite

of SPR4-infused mice relative to vehicle infused mice; **WT** = wild type mice, **HYP** = X-linked hypophosphatemic rickets mice.

Figure 3.

Bone histology and histomorphometric analysis (μ CT) of wild type and HYP mice infused with vehicle and SPR4-peptide. Mice were sacrificed on day 28 and femurs collected for analysis as described in methods. Values are means of percentage differences and are significant (* $p < 0.05$) unless indicated by NS (unpaired t test, confidence interval = 95%). Column headings represent; WT = wild type mice, HYP = X-linked hypophosphatemic rickets mice, SPR4 = infused SPR4-peptide and Vehicle = Saline infused. Histogram bars to the left of zero on the y-axis indicate down regulation and to the right up regulation: (A) Bone (femur, trabecular bone) histomorphological analysis of histology stained slides (see methods) with results and comparisons for wild type and HYP mice infused with vehicle or SPR4-peptide for 28 days. For absolute measurements in tabulated form see supplementary Table S3. Explora-Nova histomorphometric software (F17011 LaRoche Cedex 1, France) was used to analyze the microscopically imaged samples as previously described (11). The guidelines described by Ott 2008 (38) were used to calculate histomorphometric measurements. Nomenclature, symbols and units were standardized as described by Parfitt et al 1987 (37); (B) Bone (femur, epiphyseal cancellous bone) quantitative micro computed tomography (μ CT) analysis with results and comparisons for wild type and HYP mice infused with vehicle or SPR4-peptide for 28 days. For absolute measurements in tabulated form see supplementary Table S4. Micro computed tomography (μ CT) using a Scanco μ CT

40 system was carried out as described previously and discussed in methods (11). Bone histomorphometric nomenclature, symbols and units were standardized as described by Parfitt et al 1987 (37) with guidelines for assessment from Bouxsein et al 2010 (36); **(C)** Bone (femur, cortical bone) histomorphological analysis of histology stained slides (see methods) with results and comparisons for wild type and HYP mice infused with vehicle or SPR4-peptide for 28 days. For absolute measurements in tabulated form see supplementary Table S6. Explora-Nova histomorphometric software (F17011 LaRoche Cedex 1, France) was used to analyze the microscopically imaged samples as previously described (11). The guidelines described by Ott 2008 (38) were used to calculate histomorphometric measurements. Nomenclature, symbols and units were standardized as described by Parfitt et al 1987(37); **(D)** Bone (femur, cortical bone) quantitative micro computed tomography (uCT) analysis with results and comparisons for wild type and HYP mice infused with vehicle or SPR4-peptide for 28 days. For absolute measurements in tabulated form see supplementary see supplementary Table S5. Micro computed tomography (μ CT) using a Scanco μ CT 40 system was carried out as described previously and discussed in methods (11). Bone histomorphometric nomenclature, symbols and units were standardized as described by Parfitt et al 1987 (37) with guidelines for assessment from Bouxsein et al 2010 (36). **Index:** **GPA** = percentage difference in growth plate area; **GPW** = percentage difference in growth plate width; **Ob.S/BS** = osteoblast-surface/bone-surface; **O.Th** = percentage difference in osteoid thickness; **OS/BS** = percentage difference in osteoid-surface/bone-surface; **OV/BV** = percentage difference in osteoid-volume/ bone-volume; **EV** = percentage difference in eroded volume; **EV/BV** = percentage difference in eroded volume/bone-volume; **ES/BS** = percentage difference in eroded-surface/bonesurface; **Oc.N/BS** = percentage difference in osteoclast-number/bone-surface; **Oc.S/BS** = percentage difference in osteoclast-surface/bone-surface; **Tb.N** = percentage difference in trabecular number; **Tb.Sp** = percentage difference in trabecular spacing; **Tb.Th** = percentage difference in trabecular thickness; **BV/TV** = percentage difference in Bone volume/total volume; **Anisotropy** = percentage difference in anisotropic index of longitudinal/traverse tensile compressive force resistance; **SMI** = percentage difference in structural modeling index; **Connectivity** = percentage difference in the number of connected structures calculated by the Euler characteristic; **HAA** = percentage difference in mean/density of hydroxyapatite; **Ct.Th (Ring)** = percentage difference in cortical thickness of the cortical ring encompassing the central cancellous (trabecular) core of the epiphysis; **BA/TA** = percentage difference in bone-area/total-area; **TA** = percentage difference in total-area; **BA** = percentage difference in bone-area; **MVp** = percentage difference in marrow-volume of defined Region of Interest (ROI) of central diaphysis; **BS** = percentage difference in bone-surface; **BS/BV** = percentage difference in bone-surface/total-tissue-volume; **Ct.Ar** = percentage difference in cortical area; **vHA** = percentage difference in mean/density of hydroxyapatite; **TV α** = percentage difference in total-tissue-volume including marrow volume and TV δ ; **BV/TV α** = percentage difference in bone-volume/total-tissue-volume including marrow volume and TV δ ; **TV δ** = percentage difference in total tissue-volume excluding marrow-volume; **BV** = percentage difference in bone-volume; **BV/TV δ** = percentage difference in bone-volume/total-tissue- volume; **MOI** = percentage difference in moment of inertia; **Femur L** = percentage difference in femur length; **Ca.N** = percentage difference in canal number; **Ca.Ar** = percentage difference in canalicular area;; **Ct.Por** =

percentage difference in cortical porosity; **Ct.Ar (Bo)** = percentage difference in cortical area of bone; **Ct.Ar (Ost)** = percentage difference in cortical area of osteoid; **Ct.Th (Ost + Bo)** = percentage difference in average cortical thickness of osteoid and bone combined; **Ct.Th (Ost)** = percentage difference in average cortical thickness of osteoid; **Ct.Th (Bo)** = percentage difference in average cortical-thickness of bone; **OV/BV** = percentage difference in osteoid-volume/bone-volume; **TV δ** = percentage difference in total-issuе-volume excluding marrow-volume; **NS** = not significant; * = significant $P < 0.05$. Histogram bars to the left of zero on the axis indicate down regulation and to the right up regulation. See supplementary Tables S3, S4, S5 and S6 for absolute values and detailed statistics.

Figure 4.

Bone (femur, epiphyseal cancellous bone) quantitative micro computed tomography (μ CT) analysis and 3D colorimetric comparisons for wild type and HYP mice infused with vehicle or SPR4-peptide for 28 days. Mice were sacrificed on day 28 and femurs collected for analysis as described in methods. Column headings represent; WT = wild type mice, HYP = X-linked hypophosphatemic rickets mice, SPR4 = infused SPR4- peptide and Vehicle = Saline infused. For (A); WTVE and WTSPR4 and (B) HYPVE and HYPSPR4 the relative mineral densities are represented by a color scale defined in the figure and in the 3D sections. Specifically, blue is low mineral density and red high with intermediate densities shown in the color bar scales. Histogram bars to the right (C) graphically depict BV/TV (bone-volume/total-volume) and Tb.N (trabecular number). One way ANOVA plus Neuman-Keuls multiple comparison analysis was used for statistical analysis (** $p < 0.01$). Micro computed tomography (μ CT) using a Scanco μ CT 40 system was carried out as described previously and discussed in methods(11). Bone histomorphometric nomenclature, symbols and units were standardized as described by Parfitt et al 1987(37) with guidelines for assessment from Bouxsein et al 2010 (36). **Index:** Tb.N = trabecular number (1/mm); BV/TV = Bone-volume/total-volume [1]. See supplementary Table S4 for absolute values and detailed statistics.

Figure 5.

Dual Energy X-ray Absorptiometry (DEXA) values with 2-way ANOVA analyses. DEXA measurements using a Lunar PIXImus system were carried out as described previously and discussed in methods (11). Measurements are shown for mice prior to pump implantation and after sacrifice 28 days later. See also supplementary Table S8: **(A) Bone Mineral Density (BMD)**. HYPVE-mice BMD were significantly less than WTVE-mice BMD at all time-points and SPR4 peptide treatment significantly suppressed time-dependent gains in BMD for both wild-type and HYP mice. Phenotypic variation (including SPR4-treatment) was highly significant accounting for 65.87 % of the total variance ($F=217.12$, $DF_n=3$, $Df_d=67$ and $P<0.0001$). Also, time-changes were highly significant accounting for 23.6 % of the total variance ($F=233.34$, $DF_n=1$, $Df_d=67$ and $P<0.0001$). The phenotype/time *interaction* was significant accounting for 1.83 % of the total variance ($F=6.02$, $DF_n=3$, $Df_d=67$ and $P=0.0011$). **(B) Total Weight (gm)**. HYPVE-mice weight was significantly less than WTVE-mice weight at all time-points. SPR4 peptide treatment significantly decreased time-dependent gain in weight for both HYP mice (HYPSPR4) and WT mice (WTSPR4).

relative to respective vehicle groups. Phenotypic variation (including SPR4-treatment) was highly significant accounting for 64.65 % of the total variance ($F=40.6$, $DF_n=3$, $Df_d=32$ and $P<0.0001$). Also, time-changes were highly significant accounting for 13.64 % of the total variance ($F=25.69$, $DF_n=1$, $Df_d=32$ and $P<0.0001$). The phenotype/time *interaction* was significant accounting for 4.73 % of the total variance ($F=2.97$, $DF_n=3$, $Df_d=32$ and $P=0.0463$). **(C) Wild type (WT) and HYP mice percentage temporal changes** (Weight, BMC and BMD) for femurs and vertebrae as measured by Dual Energy X-ray Absorptiometry (DEXA). The mean percentage change over the 28 days for each metric was calculated and the percentage differences between the groups plotted as a histogram. Values are mean percentage differences and are significant (* = $P<0.05$) unless indicated by NS (unpaired t test confidence interval = 95%). For tabulated values and statistics see supplementary Table S8. **(D) SPR4-peptide treated mice percentage temporal changes** (Weight, BMC and BMD) for femurs and vertebrae as measured by Dual Energy X-ray Absorptiometry (DEXA). The mean percentage change over the 28 days for each metric was calculated and the percentage differences between the groups plotted as a histogram. Values are mean percentage differences and are significant (* = $P<0.05$) unless indicated by NS (unpaired t test confidence interval = 95%). For tabulated values and statistics see supplementary Table S8. **Index:** **WT** = mice infused with vehicle (0.9% physiological saline); **HYPVE** = X-linked hypophosphatemic rickets mice infused with vehicle (0.9% physiological saline); **WTSPR4** = wild type mice infused SPR4-peptide; **HYPSPR4** = X-linked hypophosphatemic rickets mice infused SPR4-peptide; **BMC (Femur)** = percentage difference in bone-mineral content changes over 4 weeks (femur); **BMD (Femur)** = percentage difference in bone-mineral density changes over 4 weeks (femur); **BMC (Vert)** = percentage difference in bone-mineral content changes over 4 weeks (vertebrae); **BMD (Vert)** = percentage difference in bone-mineral density changes over 4 weeks (vertebrae); **BMC (Total)** = percentage difference in total bone-mineral content changes over 4 weeks; **BMD (Total)** = percentage difference in total bone-mineral density changes over 4 weeks. For C and D see supplementary Table S8 for tabulated values and statistics.

DMP1 ASARM-motif Pathway

ASARM-peptide pathway

Figure 6.

The figure shows a scheme illustrating the ASARM-model and the pharmacological effects of SPR4-peptide (bimodal) in HYP and wild type mice. The numbers highlighted in the circles refer to the explanations in the following text. Interactions between DMP1, PHEX, $\alpha_5\beta_3$ integrin and ASARM-peptides occur on the osteocyte cell-surface. The interactions are, extracellular, dynamic and competitive. Arrows linking other factors represented in the cartoon illustrate positive and negative effector relationships: (1) Phosphorylated DMP1-ASARM motif is required for binding to PHEX and the RGD motif of DMP1 binds to $\alpha_5\beta_3$ integrin to form a [(PHEX)-(DMP1)-($\alpha_5\beta_3$ integrin)] trimeric complex. This interaction occurs on the cell surface of the osteocyte where PHEX and $\alpha_5\beta_3$ -integrin have an intramembranous, domain a short intracellular domain and a large extracellular domain; (2)

Formation of the [(PHEX)-(DMP1)-($\alpha_5\beta_3$ integrin)] trimeric complex initiates a signaling pathway (MAPK/Erk) that suppresses FGF23 expression; **(3)** Suppressed FGF23 results in increased serum phosphate by de-repressing renal Na-dependent phosphate transport and increasing bone mineralization; **(4)** SPR4-peptide binds to DMP1-ASARM motif and chronic administration (infusion) competitively prevents the binding of DMP1 to PHEX; **(5)** This results in increased expression of FGF23; **(6)** The increased FGF23 results in hypophosphatemia and has a negative effect on mineralization; **(7, 8 & 9)** The binding of PHEX to the DMP1-ASARM-motif is also competitively regulated by free ASARM-peptide. Specifically, free ASARM-peptide can directly bind to PHEX preventing the binding to DMP1 and thereby disrupting the [PHEX-DMP1- $\alpha_5\beta_3$ integrin] trimeric complex and increasing FGF23 expression; **(6 & 9)** This inhibits mineralization and induces hypophosphatemia by increasing FGF23; **(10 & 6)** Free ASARM-peptides can also directly inhibit renal phosphate uptake and mineralization (2, 3, 5, 8, 9, 12–14, 33, 34, 40–42). In X-linked and autosomal recessive rickets there are high levels of circulating ASARM-peptides (6, 8, 13). *In vitro* and *in vivo* (bolus and infusion) administration of ASARM-peptides and transgenic mice over expressing ASARM-peptides cause mineralization defects and hypophosphatemia (2–5, 7–14, 26, 33–35, 40–42); **(11)** The pleiotropic effects of SPR4-peptide occurs because SPR4-peptide also binds and neutralizes ASARM-peptides; **(1, 11, 12 & 13)** Thus, under conditions of ASARM depletion and/or acute bolus administration of SPR4, the formation of the trimeric complex is favored because of reduced ASARM-peptides (neutralized by SPR4); **(2 & 3)** This in turn results in reduced FGF23 expression hyperphosphatemia and increased mineralization. This model also explains the different effects of SPR4-peptide administration in wild-type and HYP mice since HYP-mice have defective PHEX and increased circulating ASARM-peptides.

Table 1

Serum and urine chemistries of wild type and HYP mice infused with vehicle and SPR4-peptide. Mice were sacrificed on day 28 and sera prepared from 16 hour fasted mice housed in metabolic cages. Column headings represent; WT = wild type mice, HYP = X-linked hypophosphatemic rickets mice, SPR4 = infused SPR4-peptide and Vehicle = Saline infused. Values are means with $\pm$ SEM (N=6). Unpaired t tests with confidence intervals of 95% were used for statistical analysis.

A Serum chemistry results & comparisons for wild type and HYP mice infused with vehicle or SPR4-peptide.									
Serum Markers	WT			HYP			WT versus HYP		
	VEHICLE (n=5)	SPR4 (n=5)	WT (% Change) Vehicle v SPR4	VEHICLE (n=9)	SPR4 (n=5)	HYP (% Change) Vehicle v SPR4	Vehicle (% Change) WT v HYP		
Alkaline Phos Tase	138.7 $\pm$ 7.3	156.5 $\pm$ 13.0 ^{b,c}	12.83	350.2 $\pm$ 64.6 ^a	477.3 $\pm$ 52.9 ^a	36.29	152.49 [*]		
Osteopontin	274.8 $\pm$ 28.3	546.3 $\pm$ 30.9 ^{abc}	98.8 [*]	525.7 $\pm$ 53.3 ^{ac}	1104.0 $\pm$ 125.9 ^{ab}	110.00 [*]	91.30 [*]		
Sclerostin	2.2 $\pm$ 0.04	1.3 $\pm$ 0.30 ^{ab}	-40.91 [*]	3.3 $\pm$ 0.01 ^{ac}	2.3 $\pm$ 0.36 ^b	-30.30 [*]	50.00 [*]		
Klotho (n=3)	208.1 $\pm$ 36.4	78 $\pm$ 23.4 ^a	-62.52 [*]	ND	ND	ND	ND		

B Urine chemistry results & comparisons for wild type and HYP mice infused with vehicle or SPR4-peptide.									
Urine Markers	WT			HYP			WT & HYP		
	VEHICLE (n=5)	SPR4 (n=5)	WT (% Change) Vehicle versus SPR4	VEHICLE (n=9)	SPR4 (n=5)	HYP (% Change) Vehicle versus SPR4	Vehicle (% Change) WT versus HYP		
Osteopontin	417.1 $\pm$ 65.1	1621.0 $\pm$ 167.5 ^{ab}	288.64 [*]	768.3 $\pm$ 110.0 ^{ac}	1887.0 $\pm$ 322.3 ^{ab}	145.61 [*]	84.20 [*]		
CTX/Creatinine	2.85 $\pm$ 0.19	3.80 $\pm$ 0.32 ^{ac}	33.33 [*]	4.15 $\pm$ 0.38 ^{ac}	6.87 $\pm$ 1.45 ^{ab}	65.54 [*]	45.61 [*]		
PYD/Creatinine	9.47 $\pm$ 0.90	9.12 $\pm$ 0.89 ^{bc}	-3.69	19.10 $\pm$ 1.38 ^a	21.80 $\pm$ 4.46 ^d	14.14	101.69 [*]		
FE Uric Acid %	6.8 $\pm$ 0.7	9.00 $\pm$ 1.4 ^{ab}	32.2 [*]	17.10 $\pm$ 3.4 ^{ac}	8.3 $\pm$ 1.8 ^b	-106.0 [*]	251.5 [*]		

Superscript letters (a, b or c) added to the calculated serum and urine values indicate:

- ^a = significantly different to WT vehicle (WT-VE) infused mice p < 0.008;
 - ^b = significantly different to HYP vehicle (HYP-VE) infused mice p < 0.002;
 - ^c = significantly different to HYP SPR4-peptide (HYP-SPR4) infused mice p < 0.02; and
- ND = Not done.

(*) The percentage changes between WT-VE versus WT-SPR4, HYP-VE versus HYP-SPR4 and WT-VE versus HYP-VE are shown in columns 3, 6 and 7 as indicated in the headings. In these columns (3, 6 and 7), the numbers that are significantly different (p < 0.01) are denoted by a superscript asterisk;

(A) Serum chemistry results and comparisons for wild type and HYP mice infused with vehicle or SPR4-peptide; and (B) Urine chemistry results and comparisons for wild type and HYP mice infused with vehicle or SPR4-peptide. **Index:** **Osteopontin** = osteopontin, bone sialoprotein (SPP1) (ng/mL); **Sclerostin** = sclerostin (SOST) ng/mL; **KLOTHO** = klotho (KL) (pg/mL); **CTX/Creatinine** = carboxy-terminal collagen crosslinks (ng/mL)/urinary-creatinine (mg/dL); **PYD/Creatinine** = hydroxylysylpyridinoline (collagen) (mmol/L)/urinary-creatinine (mg/dL); **Alkaline PhosTase** = circulating alkaline phosphatase (U/L); **FE uric Acid (%)** = fractional excretion of uric acid.

Table 2

A	mRNA	Femur gene expression (RNA) for wild type and HYP mice.									
		Bone									
		WT versus HYP					Vehicle versus SPR4				
		Vehicle		WT			HYP		WT		
		Mean	Std Error	Mean	Std Error	Mean	Mean	Std Error	Mean	Std Error	Mean
	SPP1	NS	—	—2.9*	±0.34	4.4*	—	—	—	—	±0.92
	OPG	NS	—	NS	—	NS	—	—	NS	—	—
	RANK	NS	—	NS	—	NS	—	—	NS	—	—
	RANKL	7.8*	±1.59	NS	—	NS	—	—	NS	—	—
	Cyclophilin	NS	—	NS	—	NS	—	—	NS	—	—
	ATF4	—1.8*	±0.32	NS	—	2.9*	—	—	2.9*	±0.55	—
	FOXO1	NS	—	NS	—	—1.6*	—	—	—1.6*	±0.21	—
	Cyclin D1	2.2*	±0.25	NS	—	—2.3*	—	—	—2.3*	±0.52	—
	MYC	NS	—	NS	—	—2.5*	—	—	—2.5*	±0.37	—
	CLOCK	—1.4*	±0.09	—1.5*	±0.15	2.8*	—	—	2.8*	±0.54	—
	CREB	NS	—	1.6*	±0.19	2.7	—	—	2.7	±1.10	—
	JUN-C	NS	—	—1.9*	±0.26	5.2*	—	—	5.2*	±0.76	—
	FOS	2.9*	±0.22	—2.3*	±0.32	—2.5*	—	—	—2.5*	±0.53	—
	PER-2	1.4*	±0.12	NS	—	—1.9	—	—	—1.9	±0.60	—
	MEPE	6.2*	±1.41	—3.5*	±1.01	NS	—	—	NS	—	—
	DMP1	5.2*	±1.61	NS	—	—4.4*	—	—	—4.4*	±0.65	—
	SOST	6.1*	±1.80	—3.6*	±0.96	—11.6*	—	—	—11.6*	±2.33	—
	GAPDH	—6.2*	±1.72	5.6*	±1.02	59.7*	—	—	59.7*	±17.91	—
	PHEX	N/A	N/A	NS	—	N/A	—	—	N/A	N/A	—

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B	Kidney gene expression (RNA) for wild type and HYP mice.									
	Kidney									
	WT versus HYP					Vehicle versus SPR4				
	Vehicle		WT			HYP		HYP		
mRNA	Mean	Std Error	Mean	Std Error	Mean	Std Error	Mean	Std Error	Mean	Std Error
KLOTHO	-1.4*	±0.18	-8.9*	±1.72	-2.5*	±0.31				
SPPI	NS	—	5.1*	±1.18	5.9*	±0.98				
SOST	3.2*	±0.32	-4.8*	±1.1	-4.7*	±0.74				
GAPDH	-2.0*	±0.28	2.4*	±0.89	NS	—				

Table 3

Bone (femur, cortical bone) dynamic histomorphological analysis of histology stained slides (see methods) with results and comparisons for wild type and HYP mice infused with vehicle or SPR4-peptide for 28 days. Mice were sacrificed on day 28 and femurs collected for analysis as described in methods. Column headings represent; WT = wild type mice, HYP = X-linked hypophosphatemic rickets mice, SPR4 = infused SPR4-peptide and Vehicle = Saline infused. Values are means with $\pm$ SEM (N=6).

Femur (cortical bone) dynamic histomorphological analysis: Wild type and HYP with vehicle or SPR4-peptide.									
Histology Femur (Cortical-bone)	WT			HYP			WT & HYP		
	VEHICLE (n=5)	SPR4 (n=5)	WT (% Change) Vehicle v SPR4	VEHICLE (n=9)	SPR4 (n=5)	HYP (% Change) Vehicle v SPR4	Vehicle (% Change) WT v HYP	Vehicle (% Change) WT v HYP	Vehicle (% Change) WT v HYP
BER/BS	0.71 $\pm$ 0.10	0.37 $\pm$ 0.02 ^{abc}	-47.89*	0.56 $\pm$ 0.05	0.66 $\pm$ 0.14	17.86		-21.13*	
MAR	1.43 $\pm$ 0.18	0.76 $\pm$ 0.06 ^{abc}	-46.85*	1.17 $\pm$ 0.12	1.39 $\pm$ 0.30	18.8		-18.18*	
MLT (days)	1.24 $\pm$ 0.31	3.04 $\pm$ 1.18 ^{abc}	145.16*	11.41 $\pm$ 4.81	15.22 $\pm$ 3.34 ^a	33.3.9		820.16*	
OMT	0.94 $\pm$ 0.12	1.36 $\pm$ 0.13 ^{ab}	44.68*	2.52 $\pm$ 0.43 ^a	2.01 $\pm$ 0.30 ^d	-20.24		168.09*	

Unpaired t tests with confidence intervals of 95% were used for statistical analysis. Superscript letters (a, b or c) added to the calculated values indicate;

^a = significantly different to WT vehicle (WT-VE) infused mice $p < 0.008$,

^b = significantly different to HYP vehicle (HYP-VE) infused mice $p < 0.002$ and

^c = significantly different to HYP SPR4-peptide (HYP-SPR4) infused mice $p < 0.02$.

ND= Not done.

(*) The percentage changes between WT-VE versus WT-SPR4, HYP-VE versus HYP-SPR4 and WT-VE versus HYP-VE are shown in columns 3, 6 and 7 as indicated in the headings. In these columns (3, 6 and 7), the numbers that are significantly different ($p < 0.01$) are denoted by a superscript asterisk.

Explora-Nova histomorphometric software (F17011 LaRochelle Cedex IFrance) was used to analyze the microscopically imaged samples as previously described (11). The guidelines described by Ott 2008 (38) were used to calculate histomorphometric measurements of bone turnover, mineralization and volume. Nomenclature, symbols and units were standardized as described by Parfitt et al 1987 (37) with guidelines for assessment from Boussein et al 2010 (36, 37). Index: BFR/BS = bone formation rate ($\mu\text{m}^3/\mu\text{m}^2$); MAR = mineral apposition rate ($\mu\text{m day}^{-1}$); MLT = mineralization lag time; OMT = osteoid maturation time.